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ORIGINAL ARTICLE

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Differential volatile emissions and salicylic acid levels from tobacco plants in response to different strains of *Pseudomonas syringae*

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Abstract Pathogen-induced plant responses include changes in both volatile and non-volatile secondary metabolites. To characterize the role of bacterial pathogenesis in plant volatile emissions, tobacco plants, *Nicotiana tabacum* L. K326, were inoculated with virulent, avirulent, and mutant strains of *Pseudomonas syringae*. Volatile compounds released by pathogen-inoculated tobacco plants were collected, identified, and quantified. Tobacco plants infected with the avirulent strains *P. syringae* pv. *maculicola* ES4326 (*Psm* ES4326) or pv. *tomato* DC3000 (*Pst* DC3000), emitted quantitatively different, but qualitatively similar volatile blends of (E)-β-ocimene, linalool, methyl salicylate (MeSA), indole, caryophyllene, β-elemene, α-farnesene, and two unidentified sesquiterpenes. Plants treated with the *hrcC* mutant of *Pst* DC3000 (*hrcC*, deficient in the type-III secretion system) released low levels of many of the same volatile compounds as in *Psm* ES4326- or *Pst* DC3000-infected plants, with the exception of MeSA, which occurred only in trace amounts. Interaction of the virulent pathogen *P. syringae* pv. *tabaci* (*Pstb*), with tobacco plants resulted in a different volatile blend, consisting of MeSA and two unidentified sesquiterpenes. Overall, maximum volatile emissions occurred within 36 h post-inoculation in all the treatments except for the *Pstb* infection that produced peak volatile emissions about 60 h post-inoculation. (E)-β-Ocimene was released in a diurnal pattern with the greatest emissions during the day and reduced emissions at night. Both avirulent strains, *Psm* ES4326 and *Pst* DC3000, induced accumulation of free salicylic acid (SA) within 6 h after inoculation and conjugated SA within 60 h and 36 h respectively. In contrast, SA inductions by the virulent strain *Pstb* occurred much later and conjugated SA increased slowly for a longer period of time, while the *hrcC* mutant strain did not trigger free and conjugated SA accumulations in amounts significantly different from control plants. Jasmonic acid, known to induce plant volatile emissions, was not produced in significantly higher levels in inoculated plants compared to the control plants in any treatments, indicating that induced volatile emissions from tobacco plants in response to *P. syringae* are not linked to changes in jasmonic acid.

Keywords Jasmonic acid · *Nicotiana* · *Pseudomonas* · Plant volatiles · Salicylic acid

Abbreviations HR: hypersensitive response · *hrcC*: a mutant strain of *Pst* DC3000 · JA: jasmonic acid · MeSA: methyl salicylate · *Psm* ES4326: *Pseudomonas syringae* pv. *maculicola* ES4326 · *Pst* DC3000: *P. syringae* pv. *tomato* DC3000 · *Pstb*: *P. syringae* pv. *tabaci* · *Pst* DC3661: a coronatine defective mutant strain of *Pst* DC3000 · SA: salicylic acid · SAR: systemic acquired resistance

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Introduction

Plants are constantly challenged by biotic agents such as herbivores and pathogens. Susceptibility or resistance to pathogen infection depends upon many subtle interactions between molecules produced by the plant and those produced by the pathogen. Infection of host plants by pathogens results in one of two possible outcomes: disease (compatible interaction) or resistance

(incompatible interaction). Incompatible interactions are usually associated with the appearance of necrotic flecks at sites of pathogen infection, termed the hypersensitive response (HR). In an HR, cell death is accompanied by the induction of multifaceted defense responses, including production of active oxygen species and antimicrobial compounds (phytoalexins), rapid cross-linking of cell-wall proteins, activation of several defense-related genes and, ultimately resistance to pathogens (Goodman and Novacky 1996; Hammond-Kosack and Jones 1996; He 1996). Several studies have shown that cell death associated with HR is controlled by a genetic program in the plant and requires active host participation (Dixon et al. 1994; Dangl et al. 1996; Greenberg 1997). In contrast, many compatible interactions result in slow, "normosensitive" (normal/expected sensitive response) cell death that spreads beyond the site of infection. It is not known what role, if any, host plants might play in cell death during compatible interactions.

The formation of necrotic lesions, either as a part of a local HR or as a symptom of disease, is followed by the onset of systemic acquired resistance (SAR; Ward et al. 1991; Uknes et al. 1993; Dixon et al. 1994; Hammond-Kosack and Jones 1996; Ryals et al. 1996). SAR refers to a distinct plant defense response that results in a nonspecific and long-lasting systemic resistance to a variety of pathogens. The detailed sequence of signal transduction events required for initiating and regulating the HR and SAR remains unclear; however, a mounting body of evidence indicates that salicylic acid (SA) plays a critical role in activation of multiple modes of plant defense response after pathogen attack (Sticher et al. 1997; Dempsey et al. 1999). It is well established that endogenous SA accumulates at the site of the HR in tobacco after inoculation with tobacco mosaic virus (Malamy et al. 1990; Silverman et al. 1993). Additionally, exogenous SA induces the expression of pathogenesis-related (PR) genes and decreases disease symptoms both in tobacco and *Arabidopsis* (White 1979; Uknes et al. 1992, 1993). Further evidence for the involvement of SA in disease resistance comes from transgenic tobacco and *Arabidopsis* expressing salicylate hydroxylase (NahG), which converts SA to biologically inactive catechol (Gaffney et al. 1993; Delaney et al. 1994). Plants expressing *NahG* accumulate little or no SA following pathogen infection, and have increased susceptibility to viral, fungal, and bacterial pathogens. In addition to SA, accumulated evidence shows that jasmonic acid (JA), an established wound signal, also mediates the activation of various defense responses against some microorganisms (Pieterse et al. 1998; Kenton et al. 1999; van Wees et al. 2000). Tobacco leaves infected with tobacco mosaic virus accumulate JA transiently during the temperature-dependent synchronized HR (Seo et al. 2001). Tobacco plants accumulate JA within 3 to 9 h of infection of *Pseudomonas syringae* pv. *phaseolicola* (Kenton et al. 1999). Infection of *Arabidopsis* with the fungal pathogen *Alternaria brassicicola* induced JA accumulation both in inoculated leaves and in untreated leaves of inoculated plants (Penninckx et al. 1996).

The complex array of chemical responses that plants display during pathogen attack includes the induced emissions of volatile organic compounds (Cardoza et al. 2002). In plant–insect interactions, induced plant volatile emissions have well documented roles in host recognition by many pest insects (Bernays and Chapman 1994) and also function as an indirect defence by attracting natural enemies of the insect herbivores (De Moraes et al. 1998; Röse et al. 1998; Turlings et al. 1990). Emission of volatiles from pathogen-infected plants may serve as a direct defense against pathogen infections. Several lipid-derived volatiles, including (*Z*)-3-hexenol and (*E*)-2-hexenal are released from *Phaseolus vulgaris* (L.) leaves during an HR response to *Pseudomonas syringae* pv. *phaseolicola*; and both (*E*)-2-hexenal and (*Z*)-3-hexenol are bactericidal, but at different concentrations (Croft et al. 1993). Corn-derived volatile compounds, hexanal and octanal, strongly inhibit radial growth of the fungus, *Aspergillus parasiticus* on solid culture media (Wright et al. 2000). Peanut plants infected with white mold, *Sclerotium rolfsii*, emitted a mixture of lipoxygenase products, terpenoids, indole, and methyl salicylate (MeSA), which were both quantitatively and qualitatively different from volatiles collected from healthy plants. Among these volatiles, (Z)-3-hexenyl acetate, linalool and MeSA significantly inhibited fungal growth on solid culture media (Cardoza et al. 2002).

Pseudomonas syringae is a Gram-negative plant pathogenic bacterium, which requires a type-III secretion system encoded by *hrp*/*hrc* genes to inject virulence effector proteins into host cells to cause disease in host plants and HR in nonhosts (Collmer et al. 2000). It has more than 40 pathovars on the basis of its host specificity, which have been widely used to study molecular mechanisms of host responses in *Arabidopsis* and tobacco (Century et al. 1995; Charkowski et al. 1998; Hendrickson et al. 2000; Mittler et al. 1999). In this study, we investigated the induced volatile emissions from tobacco plants in response to two avirulent strains *P. syringae* pv. *tomato* and *P. syringae* pv. *maculicola* (incompatible interactions), one virulent strain, *P. syringae* pv. *tabaci* (a compatible interaction), and the *hrcC* mutant strain of *P. syringae* pv. *tomato*, which is defective in its type-III secretion system and therefore nonpathogenic on tobacco. The nocturnal and diurnal volatile emissions of infected and control plants were examined over 7 days. In order to examine the role of the SA and JA signaling pathways in volatile induction during pathogen attack, we examined the levels of these plant hormones at different times after inoculation with the different strains of bacteria. In addition, another mutant strain of *P. syringae* pv. *tomato*, which is defective in its ability to produce coronatine (Moore et al. 1989), but has the type-III insertion system intact, was used as an initial probe of the possible role of coronatine in tobacco volatile emissions.

Materials and methods

Bacterial cultures

Pseudomonas syringae pv. *maculicola* ES4326 (*Psm* ES4326; Dong et al. 1991), *P. syringae* pv. *tomato* DC3000 (*Pst* DC3000; Whalen et al. 1991), *hrcC* mutant strain of *Pst* DC3000 (*hrcC*; Deng et al. 1998), and a coronatine-defective strain *P. syringae* pv. *tomato* DC3661 (*Pst* DC3661; Moore et al. 1989), were grown in King's medium B broth (King et al. 1954), supplemented with 50 μg/ml rifampicin for 18 h at 28 °C on a shaker at 200 rpm. *P. syringae* pv. *tabaci* (*Pstb*, Department of Plant Pathology, University of Florida, FL, USA) was grown the same way as the other strains, but with no rifampicin.

Plant material

Seeds of tobacco (*Nicotiana tabacum* L. strain K326) were sown in a commercial soil mix (MetroMix 300; Scotts-Sierra Horticultural Company, Marysville, OH, USA) and grown in an environmental chamber (E15; Control Environments, Manitoba, Canada) at 25 °C, and a relative humidity of 60–70%. Illumination with 400-W metal-halide and high-pressure sodium lamps provided a photoperiod of 12:12 h (light:dark). After 16 days, the soil was gently washed off the roots of seedlings with tap water and each seedling was transferred to a 1.0-l plastic cup containing a nutrient solution described by Baldwin and Schmelz (1994) with modifications as follows (mM): KNO_3, 1; NH_4NO_3, 0.5; $Ca(NO_3)_2.4H_2O$, 0.5; $MgSO_4.7H_2O$, 0.75; KH_2PO_4, 0.5; NaCl, 0.25; K_2SO_4, 0.25; Fe-Na-EDTA, 0.06; H_3BO_3, 0.05; $MnCl_2.4H_2O$, 0.015; $ZnSO4.7H_2O$, 0.002; $CuSO_4.5H_2O$, 0.00025; $Na_2MoO_4.2H_2O$, 0.0002. The nutrient solution in each cup was replenished after 10 days. Plants were used for experiments about 30 days after seeds were planted and had six to seven leaves including the newly emerged leaf.

Plant inoculations

One day prior to plant inoculations, the bacterial strains were cultured in King's medium B as described above. Bacterial cells were collected by centrifugation at 4,000 g for 15 min and resuspended in distilled water. The density of bacterial cell suspensions was determined as colony-forming units/ml (CFU/ml) at 600 nm with a Spectro 22 spectrophotometer (Labomed, Culver city, CA, USA) (1 $OD_{600\ nm} = 10^9$ CFU/ml) and adjusted to 4×10^7 CFU/ml supplemented with 0.04% Silwet L-77 (OSI Specialties, Friendly, WV, USA). Tobacco plants were inoculated by applying the bacterial suspension as a fine mist with a hand sprayer until the suspension ran off the leaf surfaces. Control plants were sprayed with 0.04% (v/v) Silwet L-77 in distilled water only. Plants were inoculated between 8:30 and 9:30 am. Volatiles were collected as described below.

In experiments where leaves were infiltrated to examine HR appearance, bacterial suspensions were pressure-infiltrated into the abaxial side of the leaves over an area of about one panel using a 1-ml sterilized plastic syringe without a needle. One panel refers to the fleshy region between the major veins that branch off from the mid-rib of the leaf. The HR was assessed visually as collapsed tissue at the site of infection (macroscopic HR).

Collection and analysis of volatiles

Within 1 h after inoculation, treated plants were transferred from the original hydroponic cups to glass dishes (80 mm diameter × 40 mm high) containing 130 ml of hydroponic solution. Individual plants along with the dish were then placed inside a bell-shaped glass chamber (17.8 cm high × 16.5 cm diameter at base; Analytical Research Systems, Micanopy, FL, USA). Air, purified by passage through a charcoal column entered through a hole in the base of the collection chamber at a rate of approximately 3 l/min, passed over the plants, and volatiles were sampled by pulling air from the top of the chamber at a rate of 500 ml/min through a trap containing 25 mg of Super Q adsorbent (Alltech Assoc., Deerfield, IL, USA). The remainder of the air was vented out the bottom of the system. Thus, the quantity of volatiles analyzed represents 1/6 of total plant volatile emissions. Volatiles were collected continuously, in two samples, 12 h light and 12 h dark periods, for 7 days after inoculation.

Compounds were eluted from individual traps with 150 μl methylene chloride (capillary GC/GC–MS solvent; Burdick & Jackson, Muskegon, MI, USA) and 400 ng of *n*-octane and nonyl acetate were added as internal standards. Of each sample, 1 μl was analyzed by capillary gas chromatography (Hewlett-Packard HP6890 equipped with a Hewlett-Packard 7863 auto sampler, Palo Alto, CA, USA) on an HP-1 cross-linked methyl siloxane column (15 m × 250 μm i.d., 0.25 μm film thickness) with a splitless injector at 220 °C and flame ionization detector at 250 °C. Following injection, column temperature was held at 40 °C for 30 s, then increased at 12 °C/min to 180 °C. Helium was used as a carrier gas at a flow rate of 1.2 ml/min. Data were collected with Hewlett-Packard ChemStation software and volatile compounds were quantified by comparing their peak areas with that of the internal standard, nonyl acetate. For compound identification, 1 μl of selected samples was injected into a GC, equipped with an HP-1MS column (30 m × 250 μm i.d., 0.25 μm film thickness) and interfaced to a 5973 mass selective detector (Hewlett-Packard). Mass-spectral analyses were conducted in both electron impact and chemical ionization modes. Isobutane reagent gas was used in the chemical ionization mode. The oven was held at 35 °C for 1 min, increased 10 °C/min to 230 °C, and held for 5 min. Helium was used as a carrier gas at a flow rate of 0.7 ml/min. Individual volatile components were identified by comparing their chromatographic retention times and mass spectra with those of authentic commercially available standards.

Quantitative analysis of endogenous JA and SA

Before inoculation, four plants were sampled and treated as 0 h post-inoculation. Plants inoculated with *Pst* DC3000, *Psm* ES4326 or *hrcC* mutant were sampled at 6, 12, 24, 36, 60, and 84 h post-inoculation, while plants inoculated with *Pstb* were sampled at 1, 2, 3, 4, 5, 6, and 7 days post-inoculation, because plants developed symptoms much more slowly after the infection with *Pstb* than with *Pst* DC3000 or *Psm* ES4326. Four replicates for each time point were obtained for plants under the different inoculation treatments. Plant tissues were ground under liquid nitrogen, and 0.3–0.4 g of ground tissue was added to a 15-ml falcon tube, containing dihydrojasmonic acid (500 ng) and deuterated SA (500 ng) as internal standards. The extraction and quantitative analysis of JA and SA followed the protocol of Engelberth et al. (2002).

Statistical analysis

All the data were subjected to log (x + 1) transformations since the original data did not meet the assumptions of normality and homogeneity of variance required by ANOVA. Data generated from volatile collection experiments with *Psm* ES4326, *Pst* DC3000, *hrcC*, and *Pstb* were analyzed as a 5×14 factorial design by Proc GLM (SAS Institute 1999). Bacterial strains were treated as one factor with five levels including control, and the collection time was treated as the other factor with 14 levels from 12 h to 168 h post-inoculation. Data generated from hormonal analysis were analyzed as 4×6 (for the avirulent and mutant strains, time 0 was not included) and 2×7 (for the virulent strain, time 0 was not included) factorial designs by Proc GLM after log (x + 1) transformations. Significant ANOVAs were followed by Tukey's HSD test.

Results

Volatile induction

Profiles of tobacco volatiles induced by different strains of Pseudomonas syringae Inoculation of tobacco plants by spraying with *Pst* DC3000, *Psm* ES4326, *hrcC*, *Pstb* or *Pst* DC3661 resulted in the release of a series of volatile compounds. Typical chromatograms of induced volatiles from tobacco plants after inoculation with different strains are shown in Fig. 1. Quantities of representative volatile compounds released in 12-h increments over a period of 1 week are shown in Figs. 2, 3 and 4. Visually assessed necrotic lesions first appeared at about 14 and 20 h after inoculation with *Pst* DC3000 and *Psm* ES4326, respectively. At least 12 compounds were released by *Pst* DC3000- or *Psm* ES4326-infected plants including the monoterpenes (E)-β-ocimene and linalool, the sesquiterpenes β-elemene, caryophyllene, α-farnesene, two unidentified sesquiterpenes denoted as sesq1 and sesq2, and the aromatic compounds methyl salicylate (MeSA) and indole (Fig. 1b, c). MeSA, which was one of the major compounds released from plants infected with *Psm* ES4326 or *Pst* DC3000, was not detected in plants treated with the *hrcC* mutant strain (Fig. 1d), and likewise these plants displayed no HR or disease symptoms. However, in volatiles from plants treated with the *hrcC* mutant, (E)- β-ocimene (Fig. 3) and the sesquiterpenes (Fig. 4) were detected in significantly greater amounts than from the control plants. Plants treated with coronatine-defective *Pst* DC3661 displayed HR lesions and emitted volatile blends consisting of MeSA, sesq1 and sesq2 (Fig. 1e), similar to those treated with *Pstb* (Fig. 1f), which causes wildfire disease of tobacco (necrotic spots surrounded by yellow halos). These volatiles were not detected from control plants, sprayed with surfactant alone (Fig. 1a).

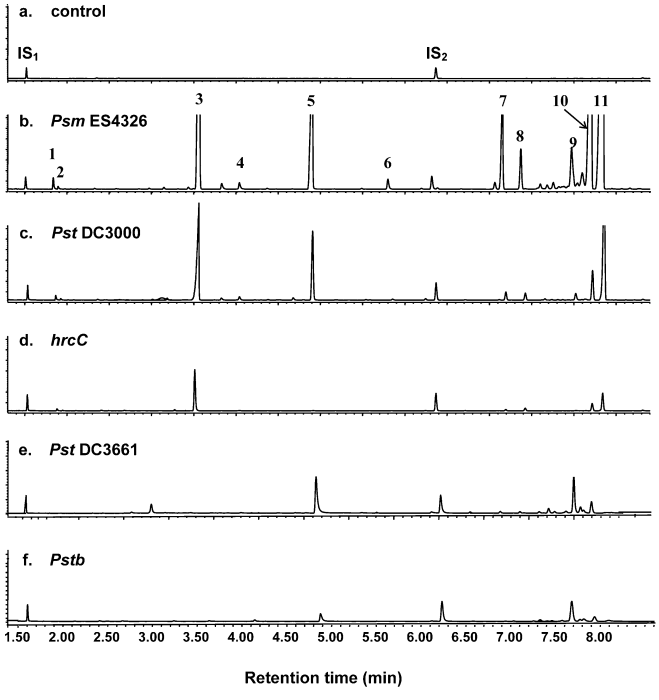

Fig. 1a–f (**a**) Typical chromatographic profiles of volatiles released by tobacco (*Nicotiana tabacum*) plants inoculated with bacterial suspensions. Volatiles shown were collected 24 h after inoculation with *Psm* ES4326 (**b**), *Pst* DC3000 (**c**), mutants *hrcC* (**d**) or *Pst* DC3661 (**e**) of *Pst* DC3000, or 48 h after inoculation with *Pstb* (**f**). Control plants (**a**) were sprayed with 0.04% (v/v) Silwet L-77 alone and volatiles collected 24 h later. The compounds represented by peaks in the chromatograms were identified as: *3*, (E)-β-ocimene; *4*, linalool; *5*, methyl salicylate; *6*, indole; *7*, β-elemene (based on comparison of mass spectra with spectra from the National Institute of Standards and Technology (1998) database); *8*, caryophyllene; *11*, α-farnesene. Compounds *1* and *2* are unidentified, compounds *9* (sesq1) and *10* (sesq2) are unidentified sesquiterpenes $C_{15}H_{24}$. *IS1*, *n*-octane; *IS2*, nonyl acetate

MeSA emissions and occurrence of lesions When considering the production of the individual components of the pathogen-induced volatiles, the most interesting aspect is the release of MeSA. It is released rapidly and in large amounts from plants inoculated with *Psm* ES4326, in lesser amounts from plants inoculated with *Pst* DC3000 followed by plants inoculated with *Pstb*, and not at all, or in trace amounts by plants inoculated with *hrcC* (Fig. 2). The emission of MeSA was detected in significant amounts within 12 h after inoculation with *Psm* ES4326 and *Pst* DC3000. Once necrotic lesions, which first became apparent 14–20 h after inoculation, completely developed around 36 h post-inoculation, MeSA emission gradually declined. Also, plants treated with *Pstb* released significantly

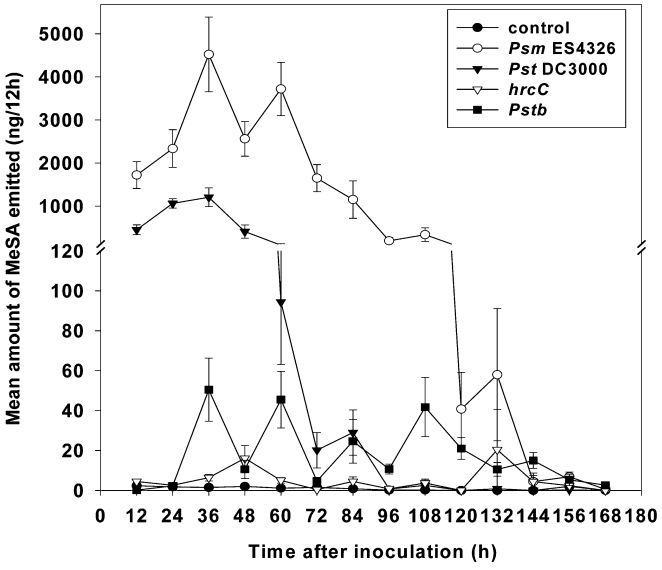

Fig. 2 Emissions of methyl salicylate (MeSA) from tobacco plants sprayed with *Psm* ES4326, *Pst* DC3000, *hrcC*, or *Pstb* at a titer of 4×10^7 CFU/ml. Volatiles were collected continuously in two samples, 12 h light and 12 h dark periods, over the collection period of 7 days. Values represent means from six replicates per strain and vertical bars indicate the SE

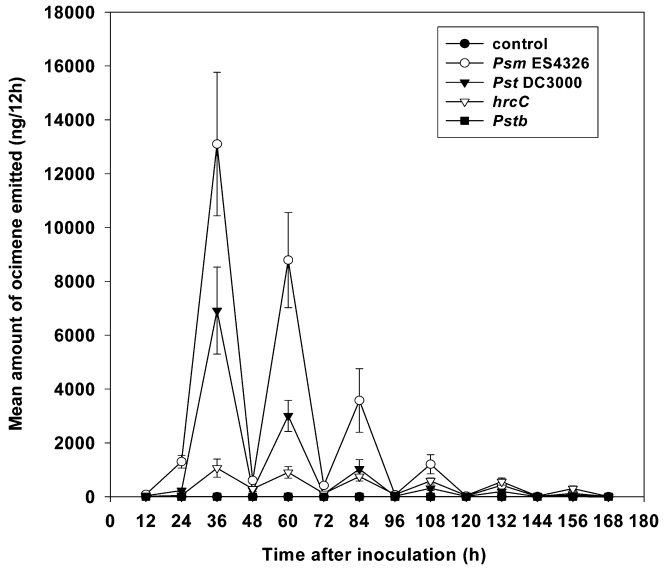

Fig. 3 Emissions of the monoterpene (E)-β-ocimene from tobacco plants sprayed with *Psm* ES4326, *Pst* DC3000, *hrcC*, or *Pstb* at a titer of 4×10^7 CFU/ml for 7 days after inoculation. Volatiles were collected in 12-h sampling periods as in Fig. 2. Values represent means from six replicates per strain and vertical bars indicate the SE

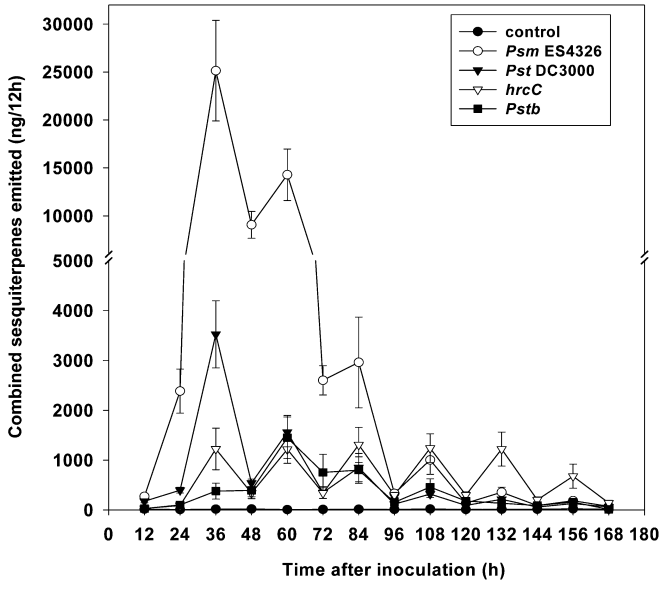

Fig. 4 Emissions of the sesquiterpenes β-elemene, caryophyllene, α-farnesene and sesq1 and 2 from tobacco plants sprayed with *Psm* ES4326, *Pst* DC3000, *hrcC*, or *Pstb* at a titer of 4×10^7 CFU/ml. Volatiles were collected in 12-h sampling periods as in Fig. 2 and amounts represent the combined total of all the sesquiterpenes. Values represent means from six replicates per strain and vertical bars indicate the SE

higher amounts of MeSA than control plants during the course of development of disease symptoms (from 36 to 108 h post-inoculation), albeit in much smaller amounts than plants treated with *Psm* ES4326 or *Pst* DC3000. There is no clear diurnal pattern of MeSA release in plants treated with *Psm* ES4326 or *Pst* DC3000. Plants inoculated with *hrcC* developed no necrotic lesions or disease symptoms and only released trace amounts of MeSA (maximum per plant of 20 ng/12 h) during the 7-day collection period.

Terpene emissions (E)-β-Ocimene was the predominant monoterpene volatile released from tobacco plants inoculated with *Psm* ES4326, *Pst* DC3000, or *hrcC*, but *Pstb* did not induce the release of ocimene in detectable amounts (Fig. 3). The ocimene emission displayed a strong diurnal pattern with maximums during the day and minimums at night. In general, the plants treated with *Psm* ES4326 released more of this compound than those treated with either *Pst* DC3000 or *hrcC*. Similarly, tobacco plants infected with *Psm* ES4326 released sesquiterpenes (β-elemene, caryophyllene, α-farnesene, sesq1, and sesq2) in the greatest amounts, followed by *Pst* DC3000-infected plants, then by *hrcC* and *Pstb*-inoculated plants (Fig. 4). Sesquiterpene release dramatically increased 12 h after inoculation and peaked during the period ending 36 h post-inoculation from plants inoculated with *Psm* ES4326 or *Pst* DC3000. However, in *Pstb*-inoculated plants, sesquiterpene emissions peaked during the period ending 60 h post-inoculation. The amounts of combined sesquiterpene emissions remained relatively steady from 24 to 144 h post-inoculation from plants inoculated with *hrcC*.

Quantitative analysis of endogenous JA and SA

All the avirulent and virulent strains of *P. syringae* induced significant increases in free and conjugated SA in the inoculated tobacco plants compared to the control plants (Fig. 5). Levels of both free and conjugated SA increased rapidly and in similar amounts in *Psm* ES4326- and *Pst* DC3000-infected plants. However, during the 12-h period ending 84 h post-inoculation, there was a statistically significant difference in the level of free SA ($P < 0.01$) in plants infected with these two strains. In contrast, although *Pstb*-infected plants accumulated both free and conjugated SA in amounts significantly higher than the controls ($P < 0.01$), the level of conjugated SA increased gradually over the entire 7 days of the experiment. Only plants treated with *hrcC* failed to accumulate significant free and conjugated SA over the duration of the experiment. In contrast to the significant changes in SA levels, no significant accumulation of JA above control levels was detected in any plants treated with bacteria.

Discussion

Each of the five strains of *P. syringae* studied induced tobacco plants to release a quantitatively and qualitatively different blend of volatile compounds. Both

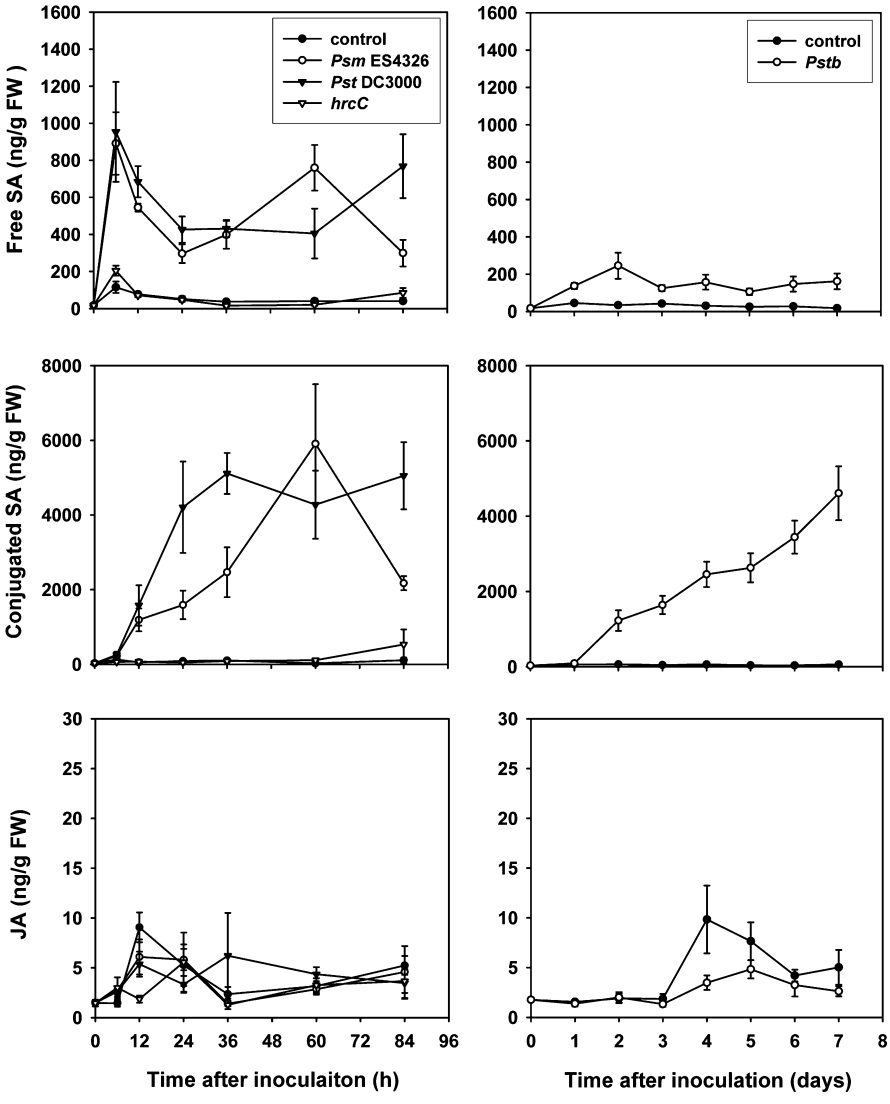

Fig. 5 Endogenous SA and JA levels following inoculation of tobacco with *Psm* ES4326, *Pst* DC3000, *hrcC*, or *Pstb* at a titer of 4×10^7 CFU/ml. Each point is the mean ± SE of at least four replicates. *FW* Fresh weight

avirulent pathogens, *Psm* ES4326 and *Pst* DC3000 elicited a very strong HR in tobacco and induced similar but complex volatile blends. In the compatible interaction, tobacco infected with *Pstb* released only small amounts of sesquiterpenes and MeSA. Furthermore, *hrcC*, which is defective in eliciting an HR or causing disease, induced release of smaller amounts of terpenes than *Psm* ES4326 or *Pst* DC3000 and almost no MeSA. Our study showed that both *Psm* ES4326 and *Pst* DC3000 induced tobacco plants to release this compound rapidly within 12 h after inoculation, that necrotic lesions first appeared 14–20 h after inoculation, and that emission of MeSA decreased after necrotic lesions developed fully (36 h post-inoculation). This result is consistent with the findings from Seskar et al. (1998) in which tobacco plants inoculated with tobacco mosaic virus and *P. syringae* pv. *phaseolicola* increased MeSA levels just before the HR-induced tissue desiccation. MeSA is believed to be synthesized from SA (Shulaev et al. 1997), which is an essential signaling element initiating SAR in plants (Gaffney et al. 1993) and is synthesized via cinnamic acid and the phenylpropanoid pathway (Lee et al. 1995). After biosynthesis, most of the free SA is removed by esterification to MeSA or conjugation to β-glucosyl-SA, which is believed to be less phytotoxic and may function as a slow-release storage form of SA that maintains SAR over extended periods of time (Lee et al. 1995; Ribnicky et al. 1998). In our study, plants treated with avirulent strains of pathogens not only released large amounts of MeSA but also accumulated very high levels of both free and conjugated SA, while plants treated with *hrcC* had almost no MeSA emissions or SA accumulations. This supports the link between free SA production and MeSA emission. Also, the absence of MeSA emission and SA accumulations in *hrcC*-treated plants suggests that factors delivered through the type-III secretion system are important for stimulation of MeSA and SA productions. It is well known that *P. syringae* requires a type-III secretion system to export and deliver virulence factors into host cells to cause pathogenesis in host plants and HR in nonhosts (Collmer et al. 2000). *Pstb* is capable of

inducing MeSA emissions and SA accumulations, but their levels were substantially lower and the inductions occurred at a later time compared to the levels induced by avirulent strains. These results suggest that tobacco plants respond with a weaker, delayed, less effective defense against *Pstb* infections.

Although plants inoculated with *hrcC* produced MeSA and SA in levels similar to controls, they released many of the other volatile compounds in significantly larger amounts than the controls. These results suggest that SA may not play a direct role in induction of volatile compounds, other than MeSA, in tobacco after inoculation of *P. syringae*. However, *P. syringae* are able to produce many toxins, which are type-III pathway-independent and generally induce a diffuse chlorosis (coronatine, phaseolotoxin, and tabtoxin; Bender et al. 1999). It has been reported that both *Psm* ES4326 and *Pst* DC3000 can produce coronatine, a phytotoxin causing leaf chlorosis and plant stunting (Cupples and Ainsworth 1995). Although the *hrcC* mutant is defective in its type-III secretion system, it is not defective in coronatine biosynthesis (Peñaloza-Vázquez et al. 2000). Coronatine, a JA mimic, is known to elicit emissions of terpenoids and other volatiles in lima bean leaves and corn plants (Koch et al. 1999; Schüler et al. 2001). In our study, plants treated with *Pst* DC3661, which is defective in coronatine production (Moore et al. 1989), released MeSA, sesq1, sesq2, and another unknown volatile compound. Thus, *Pst* DC3661-induced volatiles are very similar to *Pstb*-induced volatiles, indicating that *Pseudomonas*-derived coronatine may be responsible for ocimene and some, but not all, sesquiterpene emissions in tobacco.

Resistance against a given pathogen might also be activated via the JA signal transduction pathway or even a possible cross-talk between SA and JA pathways. For example, inoculation of *Arabidopsis* with the fungus *Alternaria brassicicola* leads to increased production of JA (Penninckx et al. 1996). Another interesting aspect of JA is its ability to induce volatile emissions (Hopke et al. 1994). JA has long been considered as an important regulator of insect-induced volatile emissions. In wild tobacco (*Nicotiana attenuata*), both JA levels and volatile emission were induced by fatty acid–amino acid conjugates present in *Manduca sexta* oral secretion (Halitschke et al. 2001). More recently, Schmelz et al. (2003) demonstrated that quantitative relationships existed between insect-induced JA levels and plant volatile emissions, and that increases in JA either preceded or paralleled increases in volatile emissions. In contrast to the literature on insect-induced volatile emissions, we found no support for a role of endogenous JA levels in the promotion of tobacco volatile emissions following pathogen attack by *P. syringae*. In a study of pathogen-induced plant responses, Stout et al. (1999) demonstrated that *P. syringae* pv. *tomato* triggers PINII gene expression in tomato during a compatible interaction. PINII gene expression is known to be highly up-regulated by increases in JA levels in tomato (Farmer et al. 1992; Conconi et al. 1996). Stout et al. (1999) did not measure endogenous levels, but did imply a role for the JA pathway in this interaction. Given our findings of induced volatile emission without changes in JA levels, we consider *P. syringae* elicitors that mimic JA signals, for example coronatine (Weiler et al. 1994; Koda et al. 1996), as probable stimuli for these responses.

To our knowledge, this is the first study in which induced volatile emissions and the plant hormones SA and JA have been studied thoroughly in interactions of plant with avirulent, virulent, and nonpathogenic strains of bacterial pathogens. The ability of tobacco plants to release not only a complex blend of volatiles compared to the healthy plants but also quantitatively and qualitatively different amounts of individual components in response to different but very closely related strains of *P. syringae* bacteria might be considered as potential criteria in identifying the causative agents of plant pathogenic infections. However, there is still much to learn about the biosynthesis and emissions of volatiles from plants in response to pathogen infection. It will be interesting to examine what, if any, *P. syringae* elicitors other than coronatine are involved in biosynthesis and release of tobacco volatiles.

Also of interest is whether volatile emission from infected plants is truly a direct defense response against pathogen infection. The correlation of volatile emissions, both temporally and quantitatively, with HR lesions, suggests that volatile emissions either play a role in the direct defense of the plants or are by-products generated from defensive responses of tobacco against *P. syringae*. Several plant volatile compounds including MeSA have antimicrobial activity in vitro (Greene-McDowelle et al. 1999; Wright et al. 2000; Cardoza et al. 2002). Therefore, it is possible that tobacco plants initiate defense responses not only by activating HR but also by releasing various volatiles against bacterial attack.

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